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Alternative Cell Line for Virus Isolation

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A human lung carcinoma cell line (A549) was compared with various other cell lines to determine susceptibility to viral growth. In the first phase of the study, A549 cells were compared with human embryonic kidney (HEK) and cynomolgus monkey kidney (CMK) cells for isolation of upper-respiratory disease viruses by using 1,248 throat swab specimens from basic-combat trainees. Of the 552 virus isolates, 507 were adenoviruses, 41 were polioviruses, and 4 were herpes simplex viruses (HSV). Of the isolates, 518 (93.8%) were isolated in A549 cells, 480 (87.0%) were isolated in HEK cells, and 262 (47.5%) were isolated in CMK cells ($P < 0.001$). In the second phase of the study, A549 cells were compared with a human diploid fibroblast cell strain (MRC-5) and Vero monkey kidney (VMK) cells for the isolation of HSV from 1,157 specimens submitted for culture. Of the 227 HSV isolates, 210 (92.5%) were isolated in A549 cells, 202 (89.0%) were isolated in VMK cells ($P > 0.1$ for A549 versus VMK cells), and 167 (73.6%) were isolated in MRC-5 cells ($P < 0.001$ for A549 versus MRC-5 cells). These results suggest that A549 cells are more susceptible to adenovirus infection and at least as susceptible to HSV infection compared with the other cell cultures evaluated. Detracting factors for the use of A549 cells were a slight loss of sensitivity to adenovirus at passage 120 and a concurrent change in the morphology of the cells. The A549 cell line proved to be an efficient, practical, and economical alternative cell system for the isolation of adenovirus and HSV in particular. Initial indications are that other clinically significant viruses may be grown in A549 cells; however, additional studies need to be performed.

In recent years, emphasis on the rapid diagnosis of viral agents has resulted in the development, improvement, and use of procedures such as immunofluorescence assays, enzyme immunoassays, and detection of viral nucleic acids by nucleic acid hybridization. Yet, with all these major advances in rapid viral identification, detection of the virus by isolation continues to be the standard by which all other methods are evaluated (13). Viral isolation procedures have high specificity, sensitivity, and versatility in detecting a wide range of viruses. However, viral isolation requires a high level of technical expertise and is often a very slow and expensive procedure. Major factors in the high cost of isolating viruses are the variety of cell cultures necessary for propagating various groups of viruses and the husbandry labor costs. Of primary concern has been the search for cell cultures that not only are sensitive but also allow optimal growth of a wide spectrum of viruses.

One of the major programs of the Virology Laboratory at Letterman Army Medical Center is the monitoring of upper-respiratory viral agents in basic-combat trainees located at various army posts across the United States. Adenovirus types 3, 4, 7, and 21 in particular cause acute respiratory disease (ARD) epidemics in military personnel (1, 5, 9) and historically have been the main focus of the program.

Over the years, we have evaluated many cell cultures for their abilities to support adenovirus growth. Cultures of continuous human epithelial-cell lines such as HeLa, KB, and HEp-2 will support adenovirus growth; however, these cells have low sensitivity for the primary isolation of adenovirus and are difficult to manage because of rapid growth and deterioration. The response of nonhuman cell culture systems to adenovirus infection has been variable, with relatively low yields of virus (7, 11). Primary human embryonic kidney (HEK) cells have consistently been the most sensitive cells for primary isolation of most adenoviruses in our laboratory. In recent years, commercial sources for HEK cells have decreased significantly, because human fetal tissue has become difficult to obtain. Additionally, the quality and sensitivity of the cells have decreased, and their cost has become prohibitive. Consequently, we have been seeking a substitute cell line with sensitivity and specificity equal to those of primary HEK cells for the isolation of adenovirus.

A human lung carcinoma continuous cell culture, A549, was first initiated by Giard et al. in 1972 (4) and further characterized by Lieber et al. in 1976 (10). These cells were derived through explant culture of lung carcinoma tissue from a 58-year-old man. Although A549 cells reportedly support viral pathogens such as adenovirus (6, 14, 15), they have not been widely accepted for general isolation purposes.

To determine the suitability of using A549 cells as a possible replacement for HEK cells in the isolation of adenovirus, we compared the sensitivity of the A549 cell culture with HEK and cynomolgus monkey kidney (CMK) cell cultures for the primary isolation of adenovirus. During our preliminary studies to determine the sensitivity of A549 cells to a variety of viruses, we observed that herpes simplex virus (HSV) grew readily in these cells. A second study was initiated to compare A549 cells with cell cultures already known to be sensitive to HSV, e.g., Vero monkey kidney (VMK) (7) and human diploid fibroblast (MRC-5) (2).

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MATERIALS AND METHODS

Test population: ARD study. A total of 1,248 throat swab specimens was collected from U.S. Army recruits at six combat training forts (Fort Benning, Ga.; Fort Jackson, S.C.; Fort Leonard Wood, Mo.; Fort Dix, N.J.; Fort Sill, Okla.; and Fort Knox, Ky.). Specimens were obtained from June through December 1983 and were submitted to the Letterman Army Medical Center Virology Laboratory. A detailed description of criteria for admission into the program, including methods of specimen collection, storage, and transport, has previously been published (3).

Test population: HSV study. A total of 1,157 specimens specifically for HSV identification was collected from military personnel, dependents, and retirees at Letterman Army Medical Center or from patients at military hospitals or clinics serviced by the Virology Laboratory. Specimens were obtained from a wide variety of sites of both genital and nongenital origin.

Cell cultures: ARD study. HEK (Microbiological Associates Bioproducts, Walkersville, Md.) and CMK (Flow Laboratories, Inc., McLean, Va.) cell culture tubes were obtained on a weekly basis. The tubes were decanted and fed with 1.5 ml of Leibovitz-15 (L-15) medium (GIBCO Laboratories, Grand Island, N.Y.) containing 2% fetal bovine serum, gentamicin (50 μg/ml), and amphotericin B (0.25 g/ml).

A549 cells were purchased from the American Type Culture Collection, Rockville, Md., (catalog no. CCL 185) at a passage level of 77. These cells were propagated in L-15 medium containing 10% fetal bovine serum, gentamicin (50 μg/ml), and amphotericin B (0.25 μg/ml). A549 cells were inoculated into 150-cm^2 flasks (Corning Glass Works, Corning, N.Y.) with 5×10^6 cells, which produced seven- to eightfold multiplication in 4 days. A549 cells (1.25×10^5 cells per tube) were inoculated into culture tubes containing L-15 medium with 2% fetal bovine serum and antibiotics as noted above.

Cell cultures: HSV study. MRC-5 cells were purchased from Microbiological Associates Bioproducts. The tubes were received weekly and fed with L-15 medium as described above. VMK cells were originally obtained from the American Type Culture Collection (catalog no. CCL 81) and were subcultured in our laboratory. Tubes of VMK cells were prepared with L-15 medium at a concentration of 1.25×10^5 cells per tube. A549 cells were obtained from the American Type Culture Collection and were prepared as described above.

Methods: ARD study. Specimens were processed and inoculated as described by Leibovitz (8). Virus isolation and identification by neutralization were performed by standard procedures (7).

Antisera for adenovirus types 3, 4, and 21 as well as poliovirus types 1, 2, and 3 were purchased from the Centers for Disease Control, Atlanta, Ga.; HSV was purchased from Microbiological Associates Bioproducts; and adenovirus 7 was purchased from Telco Laboratories, New York, N.Y. Poliovirus types 1, 2, and 3 antisera were prepared as a mixture, and the virus was identified only as polio.

To evaluate the sensitivity of A549 cells to other clinically significant viruses, we selected eight viruses for titration: HSV, coxsackievirus A21, echovirus 11, vaccinia, influenza type A Brazil, parainfluenza type 1, mumps, and cytomegalovirus. All viruses were titrated at 10^{-1} to 10^{-6}.

TABLE 1. Total number of viruses isolated and time to isolation for three cell lines

Virus	No. of isolates recovered	No. of isolates identified (avg. no. of days to CPE) with cell line		
		HEK	A549	CMK
Adenovirus type 3	3	2 (5.0)	3 (5.0)	1 (9.0)
Adenovirus type 4	276	233 (9.3)	263 (7.5)	67 (8.0)
Adenovirus type 7	187	176 (7.2)	181 (6.0)	141 (7.7)
Adenovirus type 21	41	30 (9.8)	35 (7.8)	12 (11.0)
Poliovirus	41	37 (6.5)	32 (5.0)	39 (4.0)
HSV	4	2 (11.0)	4 (7.5)	2 (15.0)
Total[a] (avg)	552	408[b] (8.1)	518[c] (6.5)	262[d] (9.1)

[a] $P < 0.001$ for A549 cells versus HEK cells and versus CMK cells (Pearson chi-square test).
[b] 87.0% positive.
[c] 93.8% positive.
[d] 47.5% positive.

Methods: HSV study. Swab specimens for HSV cultivation were received in either Virocult transport medium (Medical Wire and Equipment, Cleveland, Ohio) or tryptose phosphate broth. Two milliliters of L-15 medium containing gentamicin (100 μg/ml) and amphotericin B (0.5 μg/ml) was added to each Virocult tube. A 0.1-ml mixture of gentamicin (100 μg/ml) and amphotericin B (0.5 μg/ml) was added to each swab submitted in tryptose phosphate broth medium. Treated specimens (0.2 ml) were inoculated into one cell culture tube each of A549, MRC-5, and VMK cells. Inoculated cultures were read daily for cytopathic effect (CPE), and negative cultures were discarded at the end of 7 days. Positive isolates were identified by a modified peroxidase-antiperoxidase (PAP) method as described below. Subtyping of HSV isolates was not performed.

PAP reagents. Rabbit anti-herpes antiserum was purchased from Microbiological Associates Bioproducts. Sheep anti-rabbit immunoglobulin G (heavy- and light-chain specific) and rabbit PAP antisera were purchased from Cappel Laboratories, Cochranville, Pa. The substrate, 3-amino-9-ethylcarbizole, was purchased from Aldrich Chemical Co., San Leandro, Calif., and prepared as described by Kaplow (L. S. Kaplow, Letter, Am. J. Clin. Pathol. **63**:451, 1975). VMK cells grown on eight-chambered Lab-Tek slides were used to titrate all antisera for the PAP procedure.

PAP procedure. The procedure we used is similar to one described by Meyer et al. (12). Major differences in our procedure are the use of phosphate-buffered saline (pH 7.6) as the wash diluent, a 10-min incubation of the primary anti-herpes rabbit antibody, a 5-min incubation of the anti-rabbit immunoglobulin G and rabbit PAP, and the use of 0.05% trypan blue as a counterstain. A Dri-Bath (Thermodyne Corp., Dubuque, Iowa) placed on its side was used to incubate all tubes at 37°C.

Statistics. Specific data were analyzed by the Pearson chi-square test with a cutoff of $P = 0.05$.

RESULTS

A total of 552 virus isolates were recovered from the 1,248 throat swab specimens generated by the ARD surveillance program. The numbers and types of viruses isolated and the average number of days for a minimum of 1+ CPE to be first observed in cell culture are shown in Table 1. Of 507

TABLE 2. Spectrum of susceptibility to virus infection of four cell lines

Virus titrated	Virus passage history[a]	Virus endpoint titer for cell line			
		HEK	A549	MRC-5	CMK
HSV type 1	HEK/2, VMK/2	10^{-3}	10^{-3}	10^{-1}	NG[b]
Coxsackievirus A21	HEp/4, HEK/6	10^{-5}	10^{-3}	10^{-6}	NG
Echovirus 11	EMK/2, VMK/6	10^{-7}	10^{-5}	10^{-5}	10^{-6}
Vaccinia	VMK/4, HEK/2, VMK/1	10^{-5}	10^{-5}	10^{-4}	10^{-6}
Parainfluenza type 1[c]	RMK/5	NG	NG	10^{-1}	10^{-1}
Influenza type A Brazil[c]	RMK/4, CE al/3, RMK/2	10^{-3}	10^{-3}	10^{-1}	10^{-3}
Mumps[c]	RMK/4, VMK/3, RMK/2	10^{-1}	10^{0}	NG	10^{-2}
Cytomegalovirus	HLF/6, WI38/4, MRC/2	NG	NG	10^{-1}	NG

[a] Cell line/number of passages. HEp, human epithelial cells; EMK, embryonic monkey kidney; RMK, rhesus monkey kidney; CE al, chicken embryo allantoic fluid; HLF, human lung fibroblast; WI38, Wistar Institute (human lung fibroblast); MRC, Medical Research Center (human lung fibroblast).
[b] NG, No growth.
[c] All myxovirus titrations by hemadsorption.

adenovirus-positive specimens, 482 (95%) were positive in A549 cells, 441 (87%) were positive in HEK cells, and 221 (44%) were positive in CMK cells ($P < 0.001$). Of all the isolates, 84% were identified as adenovirus type 4 or 7. A549 cells allowed the recovery of 41 more adenovirus isolates than did HEK cells. Of all the isolates, 94% were recovered in A549 cells compared with 87% in HEK cells ($P < 0.001$). The average number of days for CPE to be observed in A549 cells for all adenoviruses was equal to or less than the time necessary in HEK and CMK cells in all cases. With only one exception, the average number of days for CPE to develop for poliovirus and HSV was less in A549 cells than in HEK or CMK cells. The combined average time before observation of CPE for all viruses isolated was at least 1.5 days earlier in A549 cells than in HEK or CMK cells.

We compiled additional data specifying the number of isolates recovered by only one cell line. A549, HEK, and CMK cells individually supported the growth of 57, 19, and 4 adenovirus isolates, respectively. If A549 cells had not been used, more than 10% of all adenoviruses would have been missed. A poliovirus also was individually recovered in A549 and CMK cells. These isolates consisted of 82 virus isolates detected by one cell line only.

Results of titrations for sensitivity showed that A549 cells were comparable to HEK, CMK, and MRC-5 cells for a wide range of viruses (Table 2) and that CMK cells were more sensitive than HEK, MRC-5, or A549 cells in the isolation of myxoviruses. Cytomegalovirus was isolated only in MRC-5 cells.

A total of 227 HSV isolates were recovered from 1,157 specimens received, for an isolation rate of 20%. The sensitivity of the cell lines plus the average number of days for a minimum of 1+ CPE to be first observed in cell culture is shown in Table 3. A549 and VMK cells showed similar sensitivity ($P > 0.1$) for the isolation of HSV and together proved to be the most sensitive combination of cells, with a sensitivity of 98.2%. The average number of days for CPE to develop was similar for all three lines. A549, VMK, and MRC-5 cells individually supported the growth of 13, 10, and 4 HSV isolates, respectively.

TABLE 3. No. of HSV isolates recovered in three cell cultures

Cell line	No. of HSV isolates identified[a]	Sensitivity (%)[b]
A549	210 (3.27)	92.5
VMK	202 (3.35)	90.0
MRC-5	167 (3.58)	73.6

[a] Number in parentheses is the average number of days to CPE. $P > 0.1$ for A549 cells versus VMK cells, and $P < 0.001$ for A549 cells versus MRC-5 cells (Pearson chi-square test).
[b] Based on a total of 227 HSV isolates.

The only detracting factors for the use of A549 cells were that a slight loss of sensitivity to adenovirus occurred at passage 120 and that a subtle degeneration of the cells took place, characterized by rounding and granulation. As a result, we limited our use of these cells to passage levels under 100 and included controls to ensure continued sensitivity to adenovirus.

DISCUSSION

During the past few years we have had difficulty obtaining good-quality cell cultures for the upper respiratory disease surveillance program. This difficulty has particularly been a problem for the primary isolation of adenovirus. Our past experience with HEK cells has shown that these cells are sensitive to a wide variety of viral pathogens, including both upper respiratory and enteric viruses. However, fewer and fewer clinical laboratories are using HEK cells for viral isolation because of prohibitive cost and lot-to-lot variability in the quality of the cells. In addition, commercially prepared cells sent through the mail are subject to improper handling, temperature variation, and shipping delays. Continuous human epithelial-cell lines such as HEp-2 and HeLa are relatively sensitive to viral isolation; however, rapid degeneration of these cell lines tends to make observation of viral CPE very difficult.

Replacing commercially prepared primary cell cultures with continuous cell lines of equal sensitivity and specificity is cost effective in any laboratory with tissue culture capabilities. Freshly prepared cells generated in our laboratory are more sensitive than commercially prepared cell cultures in tubes. Since the A549 cells can be propagated in our laboratory, their availability and sensitivity, as well as the monetary savings, are significant. The cells are easily maintained, show sustained morphology, and remain viable for 10 to 14 days in culture without significant degeneration.

Two significant observations in the ARD study were the increased sensitivity of A549 over HEK and CMK cells for primary isolation of adenovirus and the decreasing number of days necessary for viral CPE to be observed. The growth and number of days to CPE for poliovirus isolation was the only exception (Table 1). The isolation of HSV and poliovirus by A549 cells in upper respiratory disease specimens led us to believe that these cells may support the growth of a wide range of clinically significant viruses. An endpoint titration of certain viruses isolated in our labora-

tory was performed. A549 cells proved to be a sensitive cell line for those viruses titrated, with two notable exceptions (parainfluenza type 1 and cytomegalovirus) (Table 2).

In the HSV study we examined the sensitivity of A549 cells in the isolation of HSV. In our laboratory, over 80% of our clinical viral isolates are HSV. We compared A549 cell sensitivity to the two cell lines we were currently using for HSV isolation, VMK and MRC-5 cells. The increased sensitivity of A549 cells compared with that of MRC-5 cells for HSV isolation was particularly noteworthy (Table 3). A combination of A549 and VMK cells was clearly the most efficient use of cell lines for HSV isolation. By excluding MRC-5 cells from our routine procedure, we would have missed only 4 of 227 HSV isolations in this study. The average number of days to viral CPE for each line was virtually the same. In addition, results of a recent report showed that A549 cells were equally sensitive to HSV when compared with primary rabbit kidney and human foreskin fibroblast cells. (L. Sawyer and K. Kuramoto, Abstr. Int. Symp. Med. Virol., 1985, in press).

The vast majority of viruses isolated in our laboratory are HSV and adenovirus. Therefore, a cell line such as A549 with its significant sensitivity to these viruses is of great importance to us. With an increasing demand for cost efficiency but decreasing budgets, it is always important to add new technology to the laboratory that not only compares favorably with an existing technology, but also has a wide spectrum of use in similar tests. Because of our findings, we have replaced HEK cells with A549 cells in our ARD surveillance, negating the expense and variability of a commercially produced primary cell culture. For HSV specimens, A549 cells are now used routinely with only VMK cells. In addition, from January to December 1985, two vaccinia, two poliovirus type 2, eight echo 11, three varicella zoster, one coxsackievirus A21, one coxsackievirus B5, one adenovirus type 3, and one adenovirus type 6 isolate have been recovered in A549 cells from clinical specimens submitted to our laboratory.

A549 cells can now be used in those clinical microbiology laboratories that offer HSV isolation as their only in-house virology service and depend completely on commercial sources for procurement of cell lines. A549 cells are commercially available and are distributed in tubes for cell culture use in virus isolation.

We conclude that A549 human lung carcinoma cells are a cell line that is sensitive to adenovirus and HSV. Indications are that other clinically significant viruses may also be grown in these cells. However, further comparative studies should be implemented to study the potential of this cell line for practical use in various clinical virology laboratory settings.

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